Plant Archives Vol. 20, Supplement 1, 2020 pp. 2839-2844 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

INDUCTION OF *ALLIUM SATIVUM* TISSUE CULTURE BY L-METHIONINE AND GIBBERELLIC ACID AND STUDY OF THE EFFECT OF EXTRACT AGAINST FUNGAL PLANT PATHOGENS

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Abstract

Garlic is earliest known for its medicinal usage frequent components in food with significant role in disease prevention. In a plant cell and/or tissue culture study, growth regulator concentration plays a crucial role in secondary metabolite accumulation. In the present study, the antifungal activity of aqueous garlic extract was determined on representative fungi at different concentrations. The aqueous extract of garlic was obtained after the growth of garlic was induced in presence of gibberellic acid and methionine precursor. The effect of L-methionine and Gibberellic Acid was tested on yield of secondary metabolites from *Allium sativum.* Secondary metabolites of *Allium sativum* were extracted using garlic clove, leaves and shoots and quantified manually. The GC-MS analysis was used to identify solute of unknown compounds. Phenolic and flavonoid content of extracts was determined as mentioned in earlier publication. All the crude extract originated from different explants was assayed for their growth inhibition activity against *Fusarium graminearum, Fusarium oxysporum* and *Puccinia striiformis* through diffusion technique on SDA growth medium. At different time periods the antifungal activity of extracts was measured by pour plate counting method. Results showed that GC-MS analysis of the *Allium sativum* fraction had presence of 55 compounds diallyl sulfide being major bioactive fraction. Combine effect of LM and GA were significant however the individual effect of LM and/or GA was far better than combined effect on producing sulpher containing compounds. Although GA and combined effect with LM was comparable higher than but not as significant as LM on the content of phenolic and flavanoid content. *Most prominent inhibitory effect was seen on F. oxysporum* species and LM induced fraction had significant inhibition of growth. There was sharp decrease in CFU counts with LM, GA and combined treated extract. *A. sativum* are good source of antimicrobial agents can be enhanced naturally with use of elicitors and further studies can identified more potent elicitors for the plant species.

***Keyword*:** *A. sativum* ; Secondary metabolism; antimicrobial; antifungal.

Introduction

Since bacterial and fungal pathogens are exhausting with the available antimicrobial agents and rapidly evolving with resistant capability to new era drugs, the research has found its new attention to discover alternative medicinal molecules with antimicrobial activities from natural sources (Cowan, 1999). The modern era drug though more potent the over uses of it contributed in rapid growth of resistant strain worldwide. Plant based products and extracts becoming popular throughout the world for the reason it contains a variety of secondary metabolites including essential oils which are potent active towards pathogens (Taylor, 2013)

Garlic is earliest known for its medicinal usage frequent components in food with significant role in disease prevention (Chand 2013; Tessema *et al.*, 2006; EL-mahmood MA *et al.*, 2009). Literature had reported that many of the diseases can be cured with garlic consumption and it has been used against bacterial pathogens since long time. The bioactivity of the garlic lies within its secondary metabolites which is mixture of various organic and inorganic molecules (Coppi *et al.*, 2006; Wang *et al.*, 2009). Secondary metabolites are synthesized via many numbers of biosynthetic pathways in which primary metabolites concentration influences the production of the Secondary final product. These primary metabolites are often celled as effectors or precursors which stimulate production of secondary metabolites by inducing a biosynthetic pathway providing the stimulate production of secondary metabolites by inducing a biosynthetic pathway providing the initial substrate. The major classes of precursor or inducer are usually amino acids in case of plant metabolic biosynthetic pathway.

L-Methionine is one important amino acid essential not only for humans but for plant too. It is require during initiation of translation and synthesis of *S*-adenosylmethionine, the universal methylating agent. Methionine is also important in the biosynthesis of sulfur compounds, for DNA synthesis and most importantly for L-Homoserine synthesis. L-homocysteine is a toxic intermediate that interferes with ergosterol biosynthesis (Hatanaka *et al.*, 1974; McCammon and Parks 1981; Parks and Casey 1995), which is an important component of the fungal cell membrane. Inhibition of ergosterol biosynthesis is the mode of action of important antifungal drugs.

In a plant cell and/or tissue culture study, growth regulator concentration plays a crucial role in secondary metabolite accumulation. The concentration of growth hormones such as Gibberellic Acid significantly alters secondary metabolite accumulation in plant cells (Smith 1997). In the present study, the antifungal activity of aqueous garlic extract was determined on representative fungi at different concentrations. The aqueous extract of garlic was obtained after the growth of garlic was induced in presence of gibberellic acid and methionine precursor.

Materials and Methods

Reagents and chemicals

The elicitor and precursor L-methionine and Gibberellic Acid (99%, Analytical grade) was obtained from Sigma (Bagdhand, Iraq) for this present study used in the present study for treatments of *Allium sativum* culture. Other reagents were obtained from Merck, Iraq (purity ≥ 99%; analytical grade), unless not mentioned specifically in the text.

Fungi species for this study

Fusarium graminearum, Fusarium oxysporum and *Puccinia striiformis* Reference fungi, were obtained from the

fungi collection of the Plant pathology Laboratory, Mustansiriyah University. All the pathogens were cultivated in potato dextrose agar (HiMedia Laboratories, Iraq) medium under aseptic precaution using safety hood and incubated at 27 °C.

Plant material and culture establishment

Cultivars of *Allium sativum* (*A. sativum* L.) was used for the purpose. Cloves was manually, washed and air dried. The cloves were sterilized in 70% (v/v) ethanol followed by 3.5% sodium hypochlorite (10 µM Tween 80) then washed thrice with sterile distilled water. The individual cloves were inoculated containerized sterile glass containing 50 ml of half strength MS medium (Dalton *et al.,* 1983). No plant growth regulators (PGRs) were used. Sprouting plantlets as plant material were used for callus induction. The optimum culture condition supplemented with 3% (w/v) sucrose and 0.8% (w/v) and the pH of 5.8 was providing throughout growth of callus. Mass propagation was initiated conventionally for the elicitor and precursor treatment. Once there was a growth of about 5 cm, it was gradually shifted to ambient conditions to establishing for shoot expansion. All well grown and developed cultured were maintained for a couple of months in the plant tissue culture lab (Biology department, mustansiriyah university, Iraq) before elicitation process was performed using L-methionine and Gibberellic Acid. The grown callus was used to subculture into fresh MS medium once in a month for mass multiplication. One these steps are repeated for three times, callus was transferred to 50 ml of MS medium containing 3.0% (w/v) sucrose, 2.0 mg/l NAA and 1.0 mg/l kinetin.

Treatment of *Allium sativum* with L-methionine and Gibberellic Acid

The effect of L-methionine and Gibberellic Acid was evaluated and tested for its role in enhanced yield of secondary metabolites from *Allium sativum.* Stock solutions were prepared in 100 µM of 95 % of ethanol and filtered through sterilized using 0.2 µm syringe filter and stocked at 4^{o}C until required. Working solution of elicitors (10 µM) was sprayed on the grown callus of plant. Plants sprayed with distilled water were taken as a control, in this experiment. The spraying was done manually every morning sufficiently enough so that it covers plant completely it dripped and solution flows down to same soil of cultivation. This procedure was followed for 45 days and then waited for another few days until garlic was grown matured enough.

Plant extraction and estimation of secondary metabolites

Secondary metabolites of *Allium sativum* were extracted using garlic clove, leaves and shoots and quantified manually. The extractions were performed by introducing 50 g of freshly garlic, leave clover and shoots into a 1 L extractor along with 500 ml. of the series of solvent. Throughout the extraction the stirring system continued with constant stirring speed for 2 h. This extraction was subjected to sequential liquid-liquid extraction with a series of solvent including hexane, chloroform, ethyl acetate, and butanol. Then the mixture was filtered, fractions were evaporated to dryness, weighed, and stored at -4°C for further use.

Analyses of *Allium sativum* extracts: g*as chromatography-mass spectroscopy (GC-MS)*

The GC-MS analysis was performed at Central Laboratory, Biology department, Mustansiriyah University, Baghdad using a Perkin Elmer Clarus 600 gas chromatograph linked to a mass spectrometer (Turbomass). A standardized protocol was followed to run individual extract obtained from different treatment of elicitors L-methionine and Gibberellic Acid. An UV−vis detector was used to identify solute accomplished at 254 nm. The temperature was adjusted to 25 °C (isocratic), and the mobile phase flow rate was 1 mL/min, throughout the process. The GC-MS system was performed in electron ionization mode by scanning at 40 to 600 *m/z*. The injector temperature was maintained at 280 ºC. Mass spectral detection of unknown compounds were identified by comparing the spectra with those recorded in the National Institute of Standard and Technology (2005) and WILEY (2006) libraries. The total time required for analyzing a single sample was 58 minutes.

Determination of total phenolic and flavonoid content

The phenolic content was quantified following the earlier described protocol (Hidayathulla *et al.,* 2018), based on the Folin Ciocalteau method which utilizes polyphenols content gives a colored mixture with folin ciocalteau reagent and readings of the mixture are taken at 700 nm. The flavonoid content of extracts was determined as mentioned in earlier publication (method of Li *et al.,* 2007 following the steps as mentioned in Hidayathulla *et al.,* 2018).

***In vitro* antifungal activity screening using plate-hole diffusion method**

All the crude extract originated from different explants was assayed for their growth inhibition activity against *Fusarium graminearum, Fusarium oxysporum* and *Puccinia striiformis* through diffusion technique on SDA growth medium. The fungal suspension was standardized to match with Macfarland unit 1.0 (OD 595 $10^{6)}$ and 10 µL of each suspension was spread uniformly onto petri dishes agar. A 10 µL extract from each preparation was added into holes (5-mm-diameter) made on agar plate. DMSO (ethanolic solution) was used as control and Fluconazolea fungicide was used (0.1%) as positive control. Plates were incubated at 28^{0}C for 48-72 hours before measuring the diameter of the inhibition (clear zone of inhibition formed around were considered indicative of antifungal activity). Each experiment was repeated for three times and final number was average of three independent results (Jeong *et al.,* 2014).

Time-kill kinetic assay

A broth dilution method was performed to obtain an MIC value for each of extract against *Fusarium graminearum* following earlier published protocol. *F. graminearum* (1.0×10^{8} cells/ml) were treated with crude extracts at their respective MIC and incubated appropriately. At different time periods (upto 72 hours) the antifungal activity of extracts was measured by pour plate counting method (Khodavandi *et al.*, 2014), which was defined as a reduction in the number of colony forming unit (CFU)/ml, from the starting inoculum, respectively (Klepser *et al.*, 1998).

Data analysis

All experiments were conducted in a completely randomized design with three repetitions, for each treatment. The statistical analysis of the results was conducted by analysis of variance (ANOVA) statistics program 3.0 OSU Press, Ohio.

Results

The microbes used in this study were conventionally identified using biochemical's and on vitex-2 (Biomeurix, France). Results showed that GC-MS analysis of the *Allium sativum* fraction had presence of 55 compounds (Table 1). The major constituents were diallyl sulfide, methyl (E)-1-propenyl disulfide, diallyl disulfide, allyl (E)-1-propenyl

disulfide and Allyl methyl trisulfide. These major fractions were significantly increased over exposure of elicitors. All these fraction with increased production are sulpher containing compounds. Typically suplher containing compounds were efficiently produced more when influenced by L methionine compare to GA. Combine effect of LM and GA were significant however the individual effect of LM and/or GA was far better than combined effect. A number of total polyphenols and flavonoids in the extract and fractions varied with the solvent used. Interestingly there were few compounds identified in trace amount under influence of elicitors but not in normal cultivation (Table 1).

Total phenolic and flavonoid content

Ethyle acetate extract had the highest phenolic and flavanoid content. Butanol showed significantly higher phenolic and flavonoid content than the hexane and chloroform fractions. Hexane extracts showed very low concentration of phenolic compounds (Table 1). The ethyl acetate fraction showed the highest flavonoid content, followed by other extract. The exposure of LM enhanced the phenolic content as well as flavanoid content. Although GA and combined effect with LM was comparable higher than but not as significant as LM on the content of phenolic and flavanoid content (Table 2).

***In vitro* antifungal activity**

Susceptibility pattern of fungal species *Fusarium graminearum; Fusarium oxysporum; and Puccinia striiformis* to different extracts of *Allium sativum* is summarized in Table 3. The results were compared with known antifungal compound Fluconazole, and results indicated revealed that all tested extract were able to inhibit the of *fungal species. Most prominent effect was seen on Fusarium oxysporum* species and LM induced fraction had significant inhibition of growth. It was interesting to see the combined effect of LM and GA was not comparable to LM and it inhibited better than GA treated extract (Table 3).

Time-kill kinetic assay

The time-kill kinetics profile of extract of *Allium sativum* against the test organisms *Fusarium oxysporum* showed reduction in number of viable cells over the first 12, 24, 36 and 48 hours, respectively, followed by a gradual rise up to the 60 and 72 hours LM, GA and combined treatment. When compared to the control (organisms without antimicrobial agent) (Figure 1). It was interesting to see that there was sharp decrease in CFU counts upto 48 hours and thereafter there was steady growth.

Discussion

There is huge potential in natural products with novel uses in medicinal aspects, particularly related to pest management and antimicrobial. Apart from above two major applications plants are sources for metabolites which can be sued in plant disease control, contain a spectrum of secondary metabolites such as phenolics, alkaloids, flavonoids, and terpenoids. The bioactive compounds concentration in each plant species is depending upon the environmental conditions and can be enhanced with using precursor and elicitors (Shilpa 2010). Garlic extract contains a vast variety of constituents which rich source of many bioactive compounds with antimicrobial and anti fungal effects.

The presences of pathogenic microbial species are often associated with various diseases which is alarming, mainly due to the increasing rate of antibiotics resistant. Novel strategies are required to deal with increasing antimicrobial resistant and to investigate the potential activity of plants extracts for defeating the microbial drug resistance. As the plants products ad its bioactive constituents are produced very minutely and use of elicitors can be efficient to enhance the production of such active products in case they are not feasible to be synthesized chemically. This study therefore considered evaluating the antifungal potential of garlic extract and effect under influence of LM and GA elicitors. Garlic as known with its scientific name *Allium sativum* widely used in culinary and medicine (*Chiang et al.,* 2006). The therapeutic use of garlic has been recognized as a potential medicinal value to different microorganisms (*Iciek et al.,* 2005), including nosocomial *S. aureus*. This present work; explore the *in vitro* antifungal potential of *Allium sativum* extracts, against *Fusarium graminearum; Fusarium oxysporum;* and *Puccinia striiformis* and to evaluate if elicitors are able to enhanced the bioactive content of the extract.

The study showed *Allium sativum* fraction had 55 compounds of which sulfide constituents were major fractions. All sulfide fractions were significantly increased over exposure of elicitors, typically when influenced by L methionine compare to GA. Diallyl disulfide was one among major fraction which is known for its detoxication activity. Diallyl disulfide increases the glutathione S-transferase (GST) production, which plays major role in detoxication by binds electrophilic toxins in the cell (Ramage *et al.* 2005; Kuzma *et al.,* 2007; Coppi *et al.,* 2006; Gull *et al.,* 2012; Rahman *et al*., 2011; Tsao *et al.,* 2003; Hovana *et al.,* 2011). Apart from this, diallyl disulfide inhibits the growth fungal species with its oil rich content. As these bioactive contents are produced at low level, we found that contents can be enhanced with use of elicitors and results showed that exposure of LM enhanced the phenolic content as well as flavanoid content, particularly those with sulpher containing molecule included Diallyl disulfide, which was significantly increased over use of LM and GA. The time-kill kinetics profile showed reduction in number of viable fungal cells over the first 48 hours, followed by a gradual rise in growth.

Few studies have confirmed antimicrobial properties of *A. sativum* against different microorganisms; thus, the results of the present study are in agreement with other studies (Coppi *et al.,* 2006; Gull *et al.,* 2012; Rahman *et al*., 2011; Tsao *et al.,* 2003; Hovana *et al.,* 2011). Mathur *et al.* (Mathur *et al.,* 2013) showed that *A. sativum* can reduce biofilm formation of *K. pneumonia* and sandasi (Perez-Giraldo, 2003) confirmed the inhibitory effects of *A. sativum* against planktonic and biofilm formation. Although *A. sativum* extract bioactivity is studied and explored this could be first study where we have inducted the extract with use of elicitors and showed that under influence of elicitors, the bioactive compounds are enhanced and thus the activity.

Finally, we suggested that the extracts of *A. sativum* are good source of antimicrobial agents particularly against fungal pathogens and theses activity can be enhanced naturally with use of elicitors. There can be further studies to these results to further confirm garlic components possess significant bioactive properties which can be further enhanced significantly to acquire more efficient antifungal components in the extract.

Table 1 : Results showed that GC-MS analysis of the *Allium sativum* fraction had presence of 55 compounds

S. No	RI	Compound	Solvent				LM (10µM) Induced	GA (10µM)	LA (10µM) and GA (10µM)
			Butanol	Chloroform	Hexane	Ethyl acetate	Ethyl acetate		
1	837	2-Furaldehyde	0.99	1.29	2.18	1.00	2.29	2.20	2.24
2	854	(2*E*)-Hexenal	0.98	0.88	0.79	0.37	0.83	0.80	0.81
3	739	Dimethyl disulfide	1.38	0.00	0.40	0.51	0.42	0.40	0.41
4	743	2-Methylene-4-pentenal	tr	tr	0.40	0.04	0.42	0.40	0.41
5	787	3-Methylthiophene	0.10	tr	tr	0.03	tr	tr	tr
6	801	Hexanal	0.10	tr	tr	0.03	tr	tr	tr
7	842	1,2-Dithiolane	0.30	0.40	0.49	0.29	0.52	0.50	0.51
8	855	Diallyl sulfide	1.40	1.36	1.88	4.46	3.97	2.90	2.93
9	856	(3*Z*)-Hexenol	tr	tr	tr	tr	1.38	0.46	0.05
10	870	Allyl propyl sulfide	tr	tr	tr	tr	0.10	0.03	tr
11	886	Allyl (*Z*)-1-propenyl sulfide	tr	tr	tr	tr	0.03	0.02	tr
12	889	Allyl (*E*)-1-propenyl sulfide	tr	tr	tr	tr	0.01	0.04	tr
13	902	2,4-Dimethylthiophene	1.68	1.98	0.79	1.31	0.83	0.80	0.81
14	904	3,4-Dimethylthiophene	0.20	0.10	tr	0.10	tr	tr	tr
15	916	Allyl methyl disulfide	4.21	5.14	4.35	4.94	4.57	4.39	4.48
16	930	Methyl (*Z*)-1-propenyl disulfide	1.09	1.29	3.07	1.13	3.22	3.09	3.16
17	939	Methyl (*E*)-1-propenyl disulfide	3.17	2.57	2.37	3.29	12.98	12.48	12.73
18	953	1,2-Dithiolene	0.10	0.40	0.30	0.20	0.31	0.30	0.31
19	958	Benzaldehyde	0.59	4.15	0.00	1.58	0.00	0.00	0.00
20	968	Dimethyl trisulfide	2.87	1.29	1.98	1.60	2.08	2.00	2.04
21	1080	Diallyl disulfide	17.60	15.62	10.58	10.03	21.60	20.76	21.18
22	1091	Allyl (*Z*)-1-propenyl disulfide	4.25	3.36	3.07	2.88	3.22	3.09	3.16
23	1099	Allyl (*E*)-1-propenyl disulfide	10.37	7.81	10.47	8.00	12.05	11.58	11.81
24	1115	1-Propenyl propyl disulfide [d,e]	1.98	1.88	1.38	1.44	1.45	1.40	1.43
25	1123	Methyl methylthiomethyl disulfide	tr	**tr**	tr	tr	0.49	1.19	0.56
26	1135	Allyl methyl trisulfide	9.79	9.79	11.06	7.98	13.71	13.18	13.44
27	1147	4-Methyl-1,2,3-trithiolane d	1.78	0.99	0.00	0.92	0.00	0.00	0.00
28	1149	Methyl propyl trisulfide	NI	0.00	0.00	0.00	0.00	0.00	0.00
29	1158	Methyl (Z)-1-propenyl trisulfide	0.49	1.38	1.88	0.84	1.97	1.90	1.93
30	1188	3-Vinyl-4*H*-1,2-dithiine	0.79	0.59	0.89	0.56	0.93	0.90	0.92
31	1198	1,2,3-0ithia-4-cyclohexene	0.40	0.59	0.69	0.41	0.73	0.70	0.71
32	1208	Allicin	**tr**	**tr**	**tr**	**tr**	**tr**	**tr**	**tr**
33	1211	Dimethyl tetrasulfide	0.79	1.19	3.96	1.10	4.15	3.99	4.07
34	1284	Allyl methylthiomethyl disulfide[d]	2.28	2.57	0.00	1.62	**tr**	**tr**	**tr**
35	1291	Diallyl trisulfide	10.39	7.81	2.77	6.37	2.91	2.79	2.85
36	1292	Methyl (methylsulfinyl)methyl sulfide[d]	0.10	0.10	0.10	0.08	0.10	0.10	0.10
37	1301	Diallyl trisulfide	16.62	20.86	21.04	19.50	28.69	27.34	24.01
38	1302	Allyl (Z)-1-propenyl trisulfide	2.97	2.67	**tr**	1.88	**tr**	**tr**	**tr**
39	1309	*p*-Vinylguaiacol	5.14	6.43	5.24	4.44	5.50	5.29	5.40
40	1320	Allyl propyl trisulfide	0.1	tr	tr	tr	1.09	2.37	1.15
41	1325	Allyl (E)-1-propenyl trisulfide	0.2	0.18	tr	tr	**tr**	0.40	0.13
42	1344	5-Methyl-1,2,3,4-tetrathiane d	tr	tr	tr	tr	5.44	6.03	3.82
43	1346	Methyl methylthiomethyl trisulfide	0.31	0.19	0.11	0.09	0.69	0.99	0.56
44	1364	Allyl methyl tetrasulfide	1.58	2.37	2.37	1.58	2.49	2.40	2.44
45	1369	5-Methyl-1,2,3,4-tetrathiane	0.40	0.59	0.20	0.35	0.21	0.20	0.20
46	1411	1,4-Dihydro-2,3-benzoxathiin 3-oxide	0.20	0.20	0.40	0.18	0.42	0.40	0.41
47	1443	[(*E*)-1-Propenyl] 2-thiopent-3-yl disulfide [d]	0.20	**tr**	**tr**	0.07	**tr**	**tr**	**tr**
48	1483	Allyl methylthiomethyl trisulfide d	NI	NI	**tr**	tr	0.49	1.19	0.56
49	1540	Diallyl tetrasulfide	0.99	2.18	1.48	1.22	1.56	1.50	1.53
50	1591	Propyl 4-thiohept-2-en-5-yl disulfide [d]	tr	tr	tr	0.20	0.02	0.17	0.07
51	1599	Unidentified [f]	1.68	4.55	1.58	2.25	1.66	1.60	1.63
52	1646	4-Methyl-1,2,3,5,6-pentathiepane [c]	**tr**	**tr**	**tr**	**tr**	0.10	0.20	0.10
53	1754	Unidentified [g]	1.98	4.06	0.59	2.08	0.62	0.60	0.61
54	2041	Cyclooctasulfur	0.10	0.40	0.30	0.20	0.31	0.30	0.31
55	1379	Unidentified [e]	0.69	0.69	0.49	0.52	0.52	0.50	0.51

a RI = Retention index determined with respect to a homologous series of n-alkanes on an ZB-5 column.
b - = not detected. c tr =trace (<0.05%). d Identification based on MS only. L-methionine =LM;
Gibberellic Acid=GA; NI=Not identified

Table 2 : Total phenolic and flavonoid content in *Allium sativum*and its different fractions. Values are means of three replicates ± SD

Sample	Total Phenol Content as GAEmg/g of extract	Total Flavonoid Content as Quercetin mg/g of extract
Butanol fraction	28.0±2.8	18.4±1.93
Chloroform fraction	23.2±2.43	18.9±1.97
Ethyl acetate fraction	29.64±2.21	21.4±2.11
Hexane fraction	12.25±1.6	18.8±2.92
LM induced fraction in Ethyl acetate	32.1±3.8	23.2±1.92
GA induced fraction in Ethyl acetate	29.2±2.93	19.9±2.61
LA (10µM) and GA (10µM) in Ethyl acetate	29.64±2.81	20.2±2.26

L-Methionine =LM; Gibberellic Acid=GA

Table 3 : Antibacterial activity of *A sativum* extracts against fungal pathogens under influenced of elicitors and compared with control

Extracts/Antibiotic	Antibacterial activity (DIZ) in mm		
	Fusarium graminearum	*Fusarium oxysporum*	*Puccinia striiformis*
Ethyl acetate fraction	11.00±1.2^{e}	12.0±2.3^{e}	10.0±1.2^{e}
LM induced fraction in Ethyl acetate	18.0 ±2.4^{b}	20.0 ±4.3^{b}	15.0 ±3.1^{b}
GA induced fraction in Ethyl acetate	12.0 ± 0.9^{d}	15.0 ±1.6^{d}	14.0 ±2.2^{c}
LA (10µM) and GA (10µM) in Ethyl acetate	15.0 ± 1.8^{c}	18.0 ±3.5^{c}	16.0 ±.1.9^{c}
Fluconazole (0.1%)	25.0 ± 0.5^{a}	30.0 ±0.4^{a}	26.0 ±0.2^{a}

Values presented are means of six replicates, ± Standard error. The values followed by different superscript differ significantly (P<0.05). a highly significant, b significant, c least significant, d Not significant

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